AZD1208, a Potent and Selective Pan-Pim Kinase Inhibitor, Demonstrates Efficacy in Preclinical Models of Acute Myeloid Leukemia

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Running Title: AZD1208 Inhibition of Pim kinases in AML
Key Points:

- AZD1208 is a selective pan-Pim kinase inhibitor with efficacy in AML cells, xenografts and patient samples that are Flt3-ITD or Flt3 wild-type.
- AML cell growth inhibition is associated with suppression of p70S6K and 4EBP1 phosphorylation, and mRNA translation.

Abstract

Upregulation of Pim kinases is observed in several types of leukemias and lymphomas. Pim-1, -2 and -3 promote cell proliferation and survival downstream of cytokine and growth factor signaling pathways. AZD1208 is a potent, highly selective and orally available Pim kinase inhibitor that effectively inhibits all three isoforms at <5 nM or <150 nM in enzyme and cell assays, respectively. AZD1208 inhibited the growth of 5/14 AML cell lines tested and sensitivity correlates with Pim-1 expression and STAT5 activation. AZD1208 causes cell cycle arrest and apoptosis in MOLM-16 cells, accompanied by a dose-dependent reduction in phosphorylation of BAD, 4EBP1, p70S6K and S6, as well as increases in cleaved caspase 3 and p27. Inhibition of p4EBP1 and p-p70S6K, and suppression of translation, are the most representative effects of Pim inhibition in sensitive AML cell lines. AZD1208 inhibits the growth of MOLM-16 and KG-1a xenograft tumors in vivo with a clear pharmacodynamic/pharmacokinetic relationship. AZD1208 also potently inhibits colony growth, and Pim signaling substrates, in primary AML cells from bone marrow that are Flt3 wild-type or ITD mutant. These results underscore the therapeutic potential of Pim kinase inhibition for the treatment of AML.
Introduction

The Pim serine/threonine kinase family is composed of three highly homologous members, Pim-1, -2 and -3, identified as proviral insertion sites of the Moloney Murine Leukemia Virus associated with the development of T-cell lymphomas.\textsuperscript{1,2} Pim-1 and Pim-2 are upregulated in several hematological malignancies, including AML, CLL, ALL, MM and NHL.\textsuperscript{3-6} In AML, this is driven at least in part by activation of receptor tyrosine kinases such as the Flt3-ITD mutation\textsuperscript{5,7,8}, found in approximately one third of AML patients. The JAK/STAT pathway, a key mediator of cytokine and growth factor signaling, plays an important role in regulating Pim expression.\textsuperscript{9} Other pathways and transcription factors, such as NF-κB\textsuperscript{10} and HOXA9\textsuperscript{11}, may also play a role, depending on cellular context. The Pims are constitutively active kinases whose expression is controlled primarily at the transcriptional and translational level.\textsuperscript{12,13}

Pim kinases modulate the activity of a variety of substrates involved in the control of transcription, translation, cell proliferation and survival.\textsuperscript{14} They have been shown to play a role in promoting survival of AML cells via phosphorylation of Bcl-2 antagonist of cell death (BAD), abrogating its inhibitory association with anti-apoptotic protein Bcl-xL.\textsuperscript{12,15,16,17} In addition to BAD, Pims share other substrates with the AKT pathway such as PRAS40, which negatively regulates mTORC1, thereby modulating protein translation through the mTORC1 substrates p70S6K and 4EBP1.\textsuperscript{18} Evidence is accumulating for a convergent, but independent, role for Pim kinases and the AKT/mTOR pathway in the regulation of mRNA translation. Tamburini et al identified Pim-2 as an essential regulator of 4EBP1 and cap-dependent translation in AML, capable of maintaining translation in the presence of the mTORC1 inhibitor RAD001\textsuperscript{19}. Similar observations have been made in lymphoma models\textsuperscript{20}.

Inhibition of Pim kinase activity provides a novel therapeutic approach to the treatment of cancer. The attribution of oncogenic activity to all three Pim isoforms, and the potential for
redundancy, argues for the development of inhibitors capable of targeting all family members. Furthermore, gene knockout studies have demonstrated that mice deficient for all three Pims are viable and fertile\textsuperscript{21}, supportive of the tolerability of pan-Pim kinase inhibition. AZD1208 is a highly selective and orally available inhibitor of all three Pim isoforms. In this report, the efficacy of AZD1208 in cultured AML cell lines, tumor xenograft models and ex vivo cultures of primary tumor cells from Flt3-ITD and Flt3 wild-type patients is demonstrated, as well as accompanying modulation of Pim signaling substrates that can contribute to tumor growth inhibition. The results have supported the initiation of Phase I clinical trials of AZD1208 in AML.

Materials and Methods

Reagents

AZD1208 was synthesized by AstraZeneca R&D (Waltham, MA)\textsuperscript{22} and diluted in dimethyl sulfoxide (Sigma, MO). Cytarabine was purchased from Bedford Laboratories (Bedford, OH). Antibodies used are described in supplementary materials and methods.

Enzyme Assays

The activity of purified human Pim-1, -2 and -3 enzymes on a BAD peptide substrate was determined as previously described\textsuperscript{22}. To determine inhibition constants (Ki), IC\textsubscript{50} values were acquired at a series of ATP concentrations and compound doses with 1 nM enzyme and 1.5 \( \mu \)M FL-BAD substrate in 50 mM HEPES, 1 mM DTT, 0.01% Tween 20, 50 \( \mu \)g/mL BSA and 10 mM MgCl\textsubscript{2}. The Ki values were calculated by global data fitting using the Cheng-Prusoff equation or the Morrison equation for tight-binding inhibitors.
To assess selectivity, 442 kinases were screened using DiscoveRx KINOMEscan™ technology at a single concentration of 1 μM. Kinases inhibited by greater than 50% were retested at DiscoveRx with a full dose-response to determine binding affinity.

**ELISA Assay for Determination of Cellular pBAD IC50**

Details in supplementary materials and methods.

**Cell Culture and Proliferation Assay**

Cell lines were purchased from DSMZ, American Type Culture Collection or European Collection of Cell Cultures and cultured, and assayed for effect of AZD1208 on proliferation, in the recommended media as described in supplementary information.

**Cell Cycle, Cell Size and Apoptosis**

Cells were plated at 0.13 - 0.5 x 10^6 cells per well in 6 well plates overnight and treated for 72 h. For cell cycle analysis, cells were fixed in 70% ethanol before staining and treatment with propidium iodide (20 μg/mL) and RNAse A (100 μg/mL). Samples were analyzed using a BD FACS Calibur and ModFit software. For cell size measurements, the mean FSC-H parameter was determined from the G1 population using FlowJo software (Tree Star, Inc). For apoptosis, cells were stained with Annexin V (BD Bioscience) and 7AAD (BD Bioscience). Samples were analyzed on a FACS Canto using FlowJo software.

**Immunoblotting**

Cells were plated at 0.8 x 10^6 cells per well in 6 well plates overnight and then treated for the indicated times. Cell lysates were prepared using the following lysis buffers: 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40 or RIPA buffer with phosphatase inhibitor and protease inhibitor cocktails (Thermo Scientific). Protein concentration was measured using BCA reagent (Thermo Scientific) and 20-40 μg proteins were separated on Bis-Tris gels.
Immunoblotting was done with primary antibodies listed in supplementary information. After incubation with appropriate secondary antibodies, signal was detected using chemiluminescence and film or a FujiFilm Imager LAS-4000.

**Cap-dependent Translation Assay**

Cells were plated at 0.6 to 0.7 x 10^6 cells per mL in 15 mL media overnight before drug treatment for 3 h and lysis in 350 μL ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were diluted to 1 μg/μL and 450 μL was added to 55 μL methyl-7-GTP sepharose beads (GE Healthcare) and rotated overnight at 4˚C. The beads were washed and bound proteins were eluted with 1X SDS buffer, heated to 95˚C and separated on Bis-Tris 4-12% gradient gels.

**Polysome Profiling**

OCI-M1 or EOL-1 cell lines were treated with DMSO or AZD1208 (1μM) for 9 hours and polysomal fractionation was performed as described in previous studies.23,24,25

**In Vivo Studies**

5-6 week old female CB17 SCID mice purchased from Charles River Labs were maintained under specific pathogen-free conditions and used in compliance with protocols approved by the Institutional Animal Care and Use Committees of AstraZeneca, which conform to institutional and national regulatory standards on experimental animal usage.

MOLM-16 cells (5 x 10^6) or KG-1a cells (6 x 10^6) were implanted with matrigel subcutaneously into the left flank of mice. When tumor size reached ~150-200mm^3, mice were randomized and treated once daily with either vehicle (0.5% HPMC) or AZD1208 by oral gavage with 10 to 15 mice per group. Cytarabine was diluted in 0.45% NaCl and dosed
at 30mg/kg biweekly by IP injection. Tumor volume was measured twice weekly with calipers and calculated as tumor volume = \((\text{length} \times \text{width}^2) \times 0.5\).

For pharmacodynamic studies, mice with tumors of 200-400 mm\(^3\) were treated with either vehicle or AZD1208, with three mice per dose and timepoint, and tumors harvested and lysed for immunoblot analysis. Phosphorylated BAD protein levels were quantified using the phospho/total-BAD MULTI-SPOT assay system from Meso Scale Discovery (MSD).

For pharmacokinetic analysis, blood samples were collected from the same mice via cardiac puncture. Total plasma concentrations of AZD1208 were determined by LC/MS/MS method.

**Patient Samples**

Bone marrow aspirates were obtained from newly diagnosed AML patients (Dana-Farber Cancer Institute, Boston, MA) with patient consent and approval from the DFCI Institutional Review Board. This study was conducted in accordance with the Declaration of Helsinki. Mononuclear cells were isolated immediately by standard Ficoll/Paque gradient separation (Ficoll-Paque PLUS, GE Healthcare 17-1440-02) from samples delivered within 2 hours of collection. For the colony assay, cells were diluted in media and added to 3 mL of Methocult\textsuperscript{®} (StemCell Technologies #04435). AZD1208 or cytarabine was added to concentrations of 0.1, 0.3, 1 and 3 \(\mu\text{M}\) or 10 and 30 nM, respectively, and cell suspensions were mixed and plated. Following 14 days of incubation at 37\(^\circ\)C, 5% CO\(_2\) and 95% humidity, colonies were enumerated by light microscopy. Data is shown for all 15 patient samples that yielded colony growth. For biomarker analysis, cells were plated in RPMI with 2% fetal bovine serum/1% L-glutamine and treated with vehicle or 0.3, 1 and 3 \(\mu\text{M}\) AZD1208 for 3 hours. Cell lysates were prepared in Cell Lysis Buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitors.
**Results**

*AZD1208 is a potent and selective pan-Pim kinase inhibitor*

The structure of AZD1208, a benzylidene-1,3-thiazolidine-2,4-dione, is shown in Figure 1. AZD1208 is a potent ATP-competitive inhibitor of all three Pim kinase isoforms (Table 1). The inhibitory constant (Ki) values were determined to be 0.1 nM for Pim-1, 1.92 nM for Pim-2, and 0.4 nM for Pim-3. In enzymatic assays carried out at Km ATP concentrations, AZD1208 inhibited kinase activity with an IC50 of 0.4 nM for Pim-1, 5.0 nM for Pim-2 and 1.9 nM for Pim-3. In enzyme assays using 5 mM ATP, the high end of physiological ATP concentration in human cells, the IC50 values were 2.6 nM for Pim-1, 164 nM for Pim-2, and 17 nM for Pim-3.

To measure selectivity for Pims relative to other kinases AZD1208 was evaluated against a panel of 442 kinases using the DiscoveRx KINOMEScan™ competition binding assay. The three Pim kinases showed the highest percentage of inhibition. In addition, 13 other kinases were found to be inhibited by 50% or greater. No inhibition of Flt3 or Flt3 ITD was observed. A dose-response follow-up conducted against the 16 kinases inhibited >50% showed AZD1208 to bind tightly to Pim-1, -2, and -3 with binding constants (Kd) of 0.2 nM, 0.88 nM and 0.76 nM, respectively (Table 2), comparable to the inhibition constants determined in functional enzyme assays. AZD1208 bound six other kinases with potencies ranging from 38 nM to 930 nM (Table 2), but did not show significant binding to the remaining seven kinases (Kd > 10 μM). The potency difference between affinity of AZD1208 for Pim kinases and the next most potent “hit” in the panel (CDK7) was at least 43 fold.

To assess the potency of AZD1208 on each of the three Pim isoforms in a cell-based assay, a substrate-enzyme tethered system was engineered. Constructs were created with full-length Pim-1,-2 and -3 kinases individually fused to a BAD peptide substrate via a flexible (G4S)4 linker and a Myc tag at the N-terminus to allow capture in a sandwich ELISA.
fusion constructs were transiently transfected into U2-OS cells, treated with AZD1208 for 3 hours, and the level of pBAD inhibition was determined by ELISA. The IC\textsubscript{50} values were 10 nM for Pim-1, 151 nM for Pim-2 and 102 nM for Pim-3, consistent with enzyme inhibition potencies at 5 mM ATP concentration (Table 1).

**AZD1208 inhibits AML cell line growth and can induce cell cycle arrest and apoptosis**

AZD1208 was tested for anti-proliferative activity in a panel of AML cell lines (Figure 2A). Five of the cell lines tested, EOL-1, KG-1a, Kasumi-3, MV4-11 and MOLM-16, were sensitive to Pim inhibition with GI\textsubscript{50} values below 1 μM. Sensitivity in the AML panel generally correlated with high Pim-1 expression and phosphorylated STAT5. Three of the five sensitive cell lines, EOL-1, KG-1a, and MV4-11, carry known activated receptor tyrosine kinases (Table S1), and MOLM-16 cells carry a Tyk2 fusion gene (manuscript in preparation), which could account for Pim-1 upregulation downstream of STAT5 activation. MOLM-13 cells harbor the Flt3-ITD mutation and were not sensitive, but Pim-1 protein was not detected. Resistance to AZD1208 did not appear to be driven by drug efflux. Analysis of expression of 13 efflux pumps across the cell panel showed no correlation with AZD1208 GI\textsubscript{50} (R\textsuperscript{2} <0.1 for all pumps; data not shown).

Cell cycle analysis of sensitive MOLM-16 cells showed dose-dependent increases in the G0/G1 and subG1 populations, indicating both cell cycle arrest and cell death in response to AZD1208 treatment (Figure 2B). The percentage of cells in G0/G1 and subG1 increased markedly after 1 μM compound treatment for 72 h, from 54.9 to 85.7\% and 14.7 to 48.3\%, respectively. Two other sensitive cell lines tested, KG-1A and EOL-1, did not manifest cell cycle arrest in response to AZD1208, and none of the sensitive lines showed a marked apoptotic response as seen for MOLM-16 (data not shown).
Consistent with the increased subG1 cell population observed in AZD1208-treated MOLM-16 cells, a dose-dependent increase in the percentage of apoptotic and dead cells measured by Annexin V and 7AAD staining was also seen (Figure 2C). Inhibition of Pims can prevent phosphorylation of pro-apoptotic BAD leading to sequestration of the pro-survival proteins Bcl-2/Bcl-XL\textsuperscript{17}. Indeed, dose-dependent inhibition of BAD phosphorylation on Ser 112 was seen 3 h after treatment of MOLM-16 cells with AZD1208, correlated with an increase in cleaved caspase 3 (Figure 2D).

Pim kinases have also been shown to directly phosphorylate the CDK inhibitor p27, resulting in its proteosome-dependent degradation\textsuperscript{26}. Pim inhibition resulted in accumulation of p27 24 h after treatment with AZD1208 (Figure 2D), consistent with the G1 cell cycle arrest observed.

**Biomarker Modulation and Downstream Effects of AZD1208 in AML Cell Lines**

In addition to BAD, Pims have been shown to play a role in phosphorylation of other proteins shared with the PI3K/AKT/mTOR signaling pathway, as shown in Figure 3A. Modulation of several of these proteins was measured after treatment with AZD1208 in sensitive and resistant cell lines. Assessment of BAD phosphorylation on Ser 112 demonstrated inhibition by AZD1208 not only in MOLM-16 cells, but also in sensitive KG-1a, Kasumi-3, and MV4-11 cells (Figure 3B and Figure S1). However, no inhibition was observed in sensitive EOL-1 cells and varying degrees of inhibition were also seen in resistant cells, indicating that modulation of pBAD does not necessarily correlate with growth inhibition or apoptosis in these cell lines.

A better correlation with sensitivity to cell growth inhibition was seen for inhibition of phosphorylation of p70S6K on Thr 389 and its substrate the S6 ribosomal protein on Ser 235/236 (Figure 3B and Figure S1). In addition, moderate suppression of p4EBP1 on Ser 65,
most evident in samples treated with 1 uM AZD1208, was seen preferentially in sensitive cell lines. Varying degrees of inhibition of phosphorylation of the mTORC1 inhibitory protein PRAS40 on Thr 246 was seen in both sensitive (Figure 3B and Figure S1) and resistant (Figure S1) cell lines. Levels of c-myc protein were slightly reduced in some of the cell lines (Figure 3B), which could impact its transcriptional activity. In fact, modulation of the myc transcriptome was observed in cells treated with AZD1208, but in both sensitive and resistant cell lines (data not shown). No decreases were seen in cyclin D1 and Mcl-1 levels (Figure S2 and data not shown). The levels of total BAD, 4EBP1, p70S6K and S6 proteins were not reduced, although there was a slight reduction in total PRAS40 levels in two of the cell lines (Figure S2). Increases in p27 were not seen in cell lines other than MOLM-16 (data not shown).

To assess the functional consequence of inhibition of 4EBP1 phosphorylation, the effect of AZD1208 on assembly of the cap-dependent translation complex was measured. Binding of unphosphorylated 4EBP1 to the cap binding protein eIF4E blocks its interaction with eIF4G, resulting in inhibition of cap-dependent translation, whereas phosphorylation of 4EBP1 inhibits binding to eIF4E and allows its interaction with eIF4G to promote translation. In response to AZD1208 treatment, three sensitive cell lines, MOLM-16, EOL-1 and KG-1a, showed reduced interaction of immunoprecipitated eIF4E with eIF4G and increased interaction with inhibitory 4EBP1 that correlated with reduced phosphorylation of 4EBP1 on Ser 65, most evident at the 1 μM dose (Figure 3C). The effects were much less marked in AZD1208 insensitive OCI-M1 cells.

To determine whether the observed inhibition of formation of translation initiation complexes manifests at the level of mRNA translation, polysome profiling was carried out. Treatment with 1 μM AZD1208 resulted in marked suppression of polysomal peaks with a reciprocal increase in the 80S monosome peak in sensitive EOL-1 cells, but not in insensitive
OCI-M1 cells (Figure 3D), consistent with suppression of polysomal assembly preferentially in sensitive cells treated with AZD1208.

Functional deficiency of p70S6K or S6 has been linked to reduced cell size.\textsuperscript{29-32} Cell size analysis by flow cytometry demonstrated a 10 to 15% decrease in the size of MOLM-16 and KG-1a cells following treatment with AZD1208 (Figure S3), coincident with the decreases in p70S6K and S6 phosphorylation seen and comparable to that observed following treatment with rapamycin in other reports.\textsuperscript{29,30}

\textit{AZD1208 Efficacy and PK/PD in AML Xenografts}

The in vivo activity of AZD1208 was assessed in a MOLM-16 xenograft model treated daily for two weeks with drug or vehicle control. Dose-dependent inhibition of MOLM-16 tumor growth was observed (Figure 4A). Treatment with 10 mg/kg or 30 mg/kg of AZD1208 (the latter representing the typical maximum tolerated dose on this treatment schedule) led to 89% tumor growth inhibition or slight regression, respectively. Pharmacodynamic analyses following a single dose of 30 mg/kg showed strong suppression of pBAD, p4EBP1 and p-p70S6K for up to 12 hours post-dose (Figure 4B).

To determine the PK-PD relationship, inhibition of phosphorylated BAD was quantified by mesoscale ELISA in MOLM-16 tumor xenografts and plasma drug concentrations were measured following acute doses of 3, 10, and 30 mg/kg AZD1208. Dose proportional inhibition of pBAD Ser 112 was observed (Figure 4C). Following a single dose of 30 mg/kg AZD1208, BAD phosphorylation was suppressed maximally by an average of 72% for 12 h and by 46% at 24 h post-dose. At 10 mg/kg AZD1208 inhibited pBAD comparably to 30 mg/kg up to 12 h post-dose, whereas administration of 3 mg/kg showed 40 to 60% inhibition up to 9 h post-dose. Plasma drug concentrations correlated with increasing pBAD inhibition,
establishing a clear PK-PD correlation (Figure 4D). The total plasma drug concentration required to reach 50% inhibition of pBAD was 241 ng/mL, or 0.63 μM (Figure 4D).

AZD1208 was also tested in the KG-1a xenograft. Once daily treatment with 30 mg/kg resulted in 71% tumour growth inhibition at the end of the dosing period (day 39, Figure 4E). Treatment with the AML standard-of-care chemotherapeutic cytarabine (30 mg/kg twice weekly on consecutive days) resulted in 79% tumour growth inhibition. Combination of AZD1208 with cytarabine produced 96% tumor growth inhibition, and indication of a decreased rate of tumor regrowth following cessation of treatment (Figure 4E).

AZD1208 Activity in Primary AML Samples

Bone marrow aspirates obtained from newly diagnosed patients were used to evaluate the effects of AZD1208 on colony formation and Pim pathway biomarkers in primary AML cells. Mononuclear cells isolated from bone marrow aspirates were cultured in methylcellulose supplemented with cytokines and growth factors in the presence of increasing concentrations of AZD1208, cytarabine, or DMSO control for 14 days. Dose-dependent inhibition of colony growth was seen, as shown for examples in Figure 5B. Marked inhibition of colony formation (>60%) by 3 μM AZD1208 was seen in the majority of samples, including both FLT3 wild-type and ITD mutant samples, and was comparable to cytarabine in most samples (Figure 5A). Of the two samples which showed no growth inhibitory response to AZD1208, sample 020 was from a patient with acute promyelocytic leukemia, and sample 036 was uniquely resistant to colony growth inhibition by both AZD1208 and cytarabine.

Biomarker responses were also assessed in mononuclear cells treated for three hours with up to 3 μM AZD1208. The extent of inhibition of phosphorylation of BAD, 4EBP1 and p70S6K was evaluated by immunoblot analysis and summarized in Figure S4. Inhibition of
phosphorylation of 4EBP1 and p70S6K was much more prevalent than BAD, and, as was the case in the cell lines tested, showed better correlation with growth inhibition by AZD1208. Consistent with absence of Flt3 inhibition by AZD1208, suppression of pSTAT5, as seen for Pim inhibitors with Flt3 activity,\textsuperscript{33,34,35} was not observed in the Flt3-ITD patient samples (Figure S4 and data not shown).

**Discussion**

AZD1208 is an orally available Pim kinase inhibitor with an excellent selectivity profile, low nanomolar activity against all three Pim isoforms at the enzyme level and potent activity in cells (see also Dakin \textit{et al}.\textsuperscript{22}). AZD1208 inhibited growth in AML cell lines and xenograft tumor models, both as monotherapy and in combination with the standard of care cytarabine. AZD1208 also showed activity in ex vivo colony assays of progenitor cells from AML patients, where inhibition of colony growth was seen in the majority of samples tested. This included three samples with the Flt3-ITD mutation, demonstrating activity of AZD1208 in Flt-3 ITD leukemic cells. Notably, this was in the absence of the off-target inhibition of Flt3 activity characteristic of other Pim inhibitors reported to have activity in this setting\textsuperscript{33,34,35}. These results indicate Pim inhibition may be beneficial for the treatment of both Flt3 wild-type and Flt3-ITD AML.

Treatment with AZD1208 was shown to suppress Ser 112 phosphorylation of pro-apoptotic BAD, a well-documented mediator of Pim kinase pro-survival activity. In MOLM-16 cells significant, but incomplete, inhibition of pBAD was seen and correlated with induction of apoptosis. However, in AZD1208 sensitive KG-1a cells, suppression of pBAD did not correspond with increased apoptosis, and suppression of pBAD was also observed in cells insensitive to growth inhibition by AZD1208. This variability likely reflects the molecular heterogeneity of AML, the multiplicity of pathways that can contribute to BAD
phosphorylation\textsuperscript{36} and differing dependencies of the cell lines tested on BAD sequestration for cell survival.

Pim kinases have also been shown to exert more global cell growth effects by regulation of protein translation through modulation of 4EBP1 and p70S6K. Reduced phosphorylation of 4EBP1 and particularly p70S6K and its substrate S6 were the most consistent downstream effects of Pim inhibition observed in this study, and were most closely correlated with growth inhibition in the cell lines and patient samples studied. Suppression of p4EBP1 was shown to correlate with inhibition of formation of the mRNA translation initiation complex and suppression of polysome assembly, consistent with inhibition of translation. In addition, AZD1208 treatment was also associated with decreased cell size, consistent with suppression of p-p70S6K and inhibition of translation.

Although oncogenic protein synthesis through 4EBP1 was reported to be mTORC1-independent and directly controlled by Pim-2 in AML, based partly on Pim-2 siRNA studies\textsuperscript{19}, an mTORC1-dependent mechanism of Pim activity may be at least in part responsible for the inhibition of both p4EBP1 and p-p70S6K. One potential mechanism is through PRAS40, a known Pim substrate, but in our studies variable suppression of PRAS40 phosphorylation by AZD1208 was seen in both sensitive and insensitive cell lines. Alternatively, Pims could regulate mTORC1 activity in AML through upstream regulation of the mTORC1 negative regulator pTSC2 (see Figure 3A), as recently demonstrated in multiple myeloma models.\textsuperscript{37}

Ongoing preclinical and clinical analyses will be required to better understand the mechanism of action and context dependency of Pim kinase inhibition. The pan-Pim potency and selectivity of AZD1208 render it a particularly useful pharmacological tool for such studies. In addition, the activity of AZD1208 in multiple pre-clinical models of AML indicates its potential for therapeutic benefit in AML, and the ex vivo data demonstrates
activity of AZD1208 in both Flt3 WT and ITD AML primary samples. Beneficial combination with cytarabine in the tumor xenograft model supports exploration of combination of Pim inhibition with standard of care agents in AML. AZD1208 is currently in Phase 1 clinical trials.

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Authorship


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Conflicts of Interest

All authors except R.M.S., D.J.D, S.K. and L.C.P. are current or former employees of AstraZeneca.
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Table 1. Inhibition constants and enzyme assay IC₅₀ values of AZD1208

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Table 2. Binding constants for top kinase hits in the KINOMEdscan™ competition binding assay

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Figure Legends

Figure 1. Chemical structure of AZD1208.

Figure 2. Effect of AZD1208 on cell growth and survival in AML cell lines. (A) Pim 1, 2 expression and STAT3, 5 expression and phosphorylation across a panel of AML cell lines with varying sensitivity to AZD1208, as shown by immunoblot analysis of cell lysates and GI50 values from a 72 h proliferation assay. (B) AZD1208 effect on G1 cell cycle arrest and subG1 population in MOLM-16 cells, assessed by propidium iodide staining and flow cytometry for DNA content 72 h after treatment. Data shown is representative of three independent experiments. (C) Effect of AZD1208 on apoptosis in MOLM-16 cells determined by staining with Annexin V (early apoptosis), Annexin V and 7-AAD (late apoptosis) and 7-AAD (dead/necrotic cells). Data shown is representative of three independent experiments. (D) AZD1208 effect on p27, phosphorylated BAD and cleaved caspase 3, as shown by immunoblot analysis 3 and 24 h after treatment.

Figure 3. Effect of AZD1208 on downstream signaling in AML cell lines. (A) PIM signaling and interaction with PI3K/Akt/mTOR pathway showing activating (green) and inactivating (red) phosphorylation events. (B) Modulation of various biomarkers seen in MOLM-16, EOL-1, KG-1α, and OCI-M1 cells after 3 h treatment with control DMSO or 0.01, 0.1, or 1 μM AZD1208 by immunoblot analysis of cell lysates. For p70S6K the band shown represents the ~60 kDa S6K1 isoform. (C) Effect of AZD1208 on cap-dependent translation complex formation after 3 h treatment with AZD1208 at the indicated doses. eIF4E was immunoprecipitated from treated cell lysates using methyl-7 GTP cap sepharose beads (the eluted immunoprecipitated material is shown in the third row) and immunoblotted with eIF4G (first row) and 4EBP1 (second row) to assess the extent of association of each
protein with eIF4E before and after treatment with AZD1208. The fourth row shows effects of drug treatment on p4EBP1 S65 prior to immunoprecipitation of cell lysates. (D) Effects of PIM kinase inhibition on polysomal assembly. OCI-M1 or EOL-1 cell lines were treated with solvent control (DMSO), or 1 μM AZD1208 for 9 hours and the lysates were layered on a 10-50% sucrose gradient. The gradients were subjected to ultracentrifugation and fractions were collected by continuous monitoring of OD at 254nm. The OD 254nm was plotted as a function of gradient depth for each treatment. A representative profile from one out of two studies is shown and the polysomal (PS) and monosomal (MS) peaks are indicated. The area under the polysome and and monosome peaks was quantified for each treatment using Image J software. The ratio of area under the polysomal and polysomal plus monosomal peaks was calculated for each treatment and the results of two independent studies for each cell line is presented as percentage of respective DMSO control.

Figure 4. AZD1208 efficacy and pharmacokinetic/pharmacodynamic analyses in AML xenograft models. (A) CB17 SCID mice implanted subcutaneously with MOLM-16 cells were treated once daily (QD) with either vehicle or AZD1208 by oral gavage for 14 days to assess effect on tumor growth. Data shown are the average +/- SEM. **Significant difference in tumor size at day 25 between vehicle and AZD1208-treated mice, p<0.001; Student t test. (B) Immunoblot analysis of MOLM-16 tumors from mice treated with 30 mg/kg AZD1208 at the times shown. (C) Phosphorylated BAD levels as percent of vehicle-treated controls measured at the indicated times and doses, as described in methods. Data represent the average of triplicates +/- SEM. (D) Percent pBAD inhibition at multiple plasma drug levels was measured to determine the PK-PD relationship and concentration required for 50% inhibition. Data are from the study in (C) and an additional dataset including 18 h timepoints. The graph was generated using Phoenix WinNonlin 6.3 software (Pharsight). (E) CB17 SCID mice
implanted subcutaneously with KG-1a cells were treated once daily with vehicle or AZD1208 by oral gavage or twice weekly with cytarabine by IP injection from day 14 to 39 post-implantation. Data shown are the average +/- SEM. **Significant difference in tumor size at both days 39 and 50 between AZD1208-treated or cytarabine treated mice and mice treated with the combination of AZD1208 and cytarabine, p<0.001; Student t test.

**Figure 5. Inhibition of primary AML colony growth.** (A) Summary of percent inhibition of colony growth of primary marrow mononuclear cells from AML patients treated with AZD1208 or cytarabine. Data shown are from cells continuously treated for 14 days with 3 μM AZD1208 or 30 nM cytarabine. (B) Result of colony growth inhibition in Flt3 wild-type and Flt3-ITD patient samples treated with vehicle control, 0.1, 0.3, 1 and 3 μM AZD1208 in a 14 day methylcellulose assay.
Figure 1

Chemical Formula: $C_{21}H_{21}N_3O_2S$
Molecular Weight: 379.48
Figure 2

A) Western blot analysis showing expression levels of various proteins in different cell lines treated with different concentrations of AZD1208. The proteins analyzed include Pim-1, Pim-2, pSTAT5 (Y694), STAT5, pSTAT3 (Y705), STAT3, GAPDH, and AZD1208 GI50 (µM) concentrations for each cell line.

B) Flow cytometry analysis showing the cell cycle distribution under control conditions and after treatment with 1 µM AZD1208. The table below summarizes the percentage of cells in sub-G1, G0/G1, S, and G2/M phases for control and treated samples.

C) Apoptosis assay showing the percentage of cells in early, late apoptosis, and dead states at 3 hours post-treatment with different concentrations of AZD1208.

D) Western blot analysis of protein expression at 3 hours post-treatment with DMSO and various concentrations of AZD1208. The proteins analyzed include pBAD S112, Cleaved Caspase 3, Tubulin, p27, and Tubulin at 24 hours post-treatment.

<table>
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<th>AZD1208 µM</th>
<th>% sub-G1</th>
<th>% G0/G1</th>
<th>% S</th>
<th>% G2/M</th>
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</table>

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Figure 3
Figure 4

A. Tumor volume (mm$^3$) vs. Days Post Implant for different treatment groups:
- Vehicle
- AZD1208 - 0.3 mg/kg QD
- AZD1208 - 1 mg/kg QD
- AZD1208 - 3 mg/kg QD
- AZD1208 - 10 mg/kg QD
- AZD1208 - 30 mg/kg QD

B. Western blot analysis showing:
- p-BAD S112
- p-4EBP1 S65
- p-p70S6K T389
- GAPDH

C. pBAD expression as % of control over time after treatment (h):
- 3 mg/kg AZD1208
- 10 mg/kg AZD1208
- 30 mg/kg AZD1208

D. IC$_{50}$ = 241 ng/mL

E. Tumor volume (mm$^3$) vs. Days Post Implant for different groups:
- Vehicle
- Cytarabine - 30 mg/kg QDx2/wk
- AZD1208 - 30 mg/kg QD
- Cytarabine + AZD1208

**/** indicates statistical significance.
AZD1208, a potent and selective pan-pim kinase inhibitor, demonstrates efficacy in preclinical models of acute myeloid leukemia


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